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Hypoplastic Left Heart Syndrome Sequencing Reveals a Novel *NOTCH1* Mutation in a Family with Single Ventricle Defects

Matthew D. Durbin[1], **Adrian G. Cadar**[2], **Charles H. Williams**[2], **Yan Guo**[3], **David P. Bichell**[4], **Yan Ru Su**[2], and **Charles C. Hong**[2,5]

[1]Department of Pediatrics – Division of Neonatal-Perinatal Medicine, Indiana University School of Medicine, Indianapolis, IN 46202

[2]Departments of Medicine – Division of Cardiovascular Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

[3]Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

[4]Department of Pediatric Cardiac Surgery, Vanderbilt University School of Medicine, Nashville, TN 37232 USA

[5]Research Medicine, Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN 37212

Abstract

Background—Hypoplastic left heart syndrome (HLHS) has been associated with germline mutations in 12 candidate genes and a recurrent somatic mutation in *HAND1* gene. Using targeted and whole exome sequencing (WES) of heart tissue samples from HLHS patients, we sought to estimate the prevalence of somatic and germline mutations associated with HLHS.

Methods—We performed Sanger sequencing of the *HAND1* gene on 14 ventricular (9 LV and 5 RV) samples obtained from HLHS patients, and WES of 4 LV, 2 aortic and 4 matched PBMC samples, analyzing for sequence discrepancy. We also screened for mutations in the 12 candidate genes implicated in HLHS.

Results—We found no somatic mutations in our HLHS cohort. However, we detected a novel germline frameshift/stop-gain mutation in *NOTCH1* in a HLHS patient with a family history of both HLHS and hypoplastic right heart syndrome (HRHS).

Conclusion—Our study, involving one of the first familial cases of single ventricle defects linked to a specific mutation, strengthens the association of *NOTCH1* mutations with HLHS, and suggests that the two morphologically distinct single ventricle conditions, HLHS and HRHS, may share a common molecular and cellular etiology. Finally, somatic mutations in the LV are an unlikely contributor to HLHS.

*Co-corresponding authors: Charles C. Hong, MD, PhD, Vanderbilt University School of Medicine, 2220 Pierce Ave., PRB 383, Nashville, TN 37232, Telephone: (615) 936-7032, Fax: (615) 936-1872, charles.c.hong@vanderbilt.edu.

Keywords

hypoplastic left heart syndrome; congenital heart disease; whole exome sequencing; somatic mutation; HAND1 and NOTCH1

Introduction

Hypoplastic left heart syndrome (HLHS) affects 1 of every 4,344 newborns and is one of the most devastating forms of congenital heart disease (CHD) [1]. In HLHS, the left ventricle (LV) is severely underdeveloped, forming a vestigial chamber. HLHS is uniformly fatal without multi-stage palliative surgeries or cardiac transplantation [2,3].

The precise pathogenesis of HLHS is unknown, but evidence supports a genetic etiology [4–6]. HLHS is highly heritable, with a 500-fold increased incidence among siblings and a 1000-fold increase if a parent has any form of CHD [6]. Moreover, human genetic studies demonstrate a linkage to chromosome 2p15 [7], and a large scale sequencing study recently implicated the role of *de novo* mutations in HLHS, although it did not establish a definitive causality [5]. In addition, mutations in several genes are associated with HLHS, including *FOXC2*[8], *FOXL1*[8], *GJA1*[9], *HAND1*[10], *IRX4*[11], *MYH6*[12], *NKX2.5*[13], *NOTCH1*[14, 15] and *TBX5*[16].

Somatic mutations are implicated in atrial fibrillation and in various CHD, including HLHS. Reamon-Buettner and colleagues demonstrated a somatic mutation in the *HAND1* gene, confined to the hypoplastic left ventricle, but absent from adjacent tissue and peripheral blood, in HLHS patients [17]. They found 24 of 31 ventricular samples had a recurrent frameshift mutation (A126fs) in the basic helix- loop-helix (bHLH) DNA binding domain of *HAND1*. Evidence refuting this finding was recently reported [18–20], but this study analyzed only the right ventricle, which is not the primary chamber affected in HLHS, leaving open the crucial possibility that a somatic mutation restricted to the developing left ventricle resulted in HLHS. Lastly, no study has looked for somatic mutations in HLHS beyond the *HAND1* gene.

Recently, germline mutations in *NOTCH1* and several other genes have been implicated in HLHS. Garg and colleagues found that frameshift and nonsense mutations in *NOTCH1* are strongly associated with a spectrum of developmental aortic valve anomalies, providing the first human genetic evidence that *NOTCH1* mutations cause CHD [14]. Interestingly, some patients with *NOTCH1* mutations had other CHDs, including one HLHS case. Subsequently, 2 HLHS patients were found to carry *de novo* mutations in *NOTCH1*[15], and another was found to be compound heterozygous for novel missense *NOTCH1* variants [21]. Finally, in a study of 428 CHD proband, gross *NOTCH1* mutations (splicing mutations, truncations and whole gene deletion) were found in 7% of familial L-CHD and 1% of sporadic L-CHD [22]. Interestingly, only 2 of 63 families with *NOTCH1* mutation had a child with HLHS, suggesting *NOTCH1* mutations causing HLHS may be rare.

We sought to test the hypothesis that mutations, and possibly somatic mutations in the developing LV, may contribute to HLHS pathogenesis. To identify mutations, we performed

both targeted and whole exome sequencing (WES) of DNA isolated from LV tissue, the corresponding aortic outflow tract (OFT), and the peripheral blood mononuclear cells (PBMCs) obtained from HLHS patients.

Methods

Source of the tissue samples and blood and DNA isolation

We acquired tissue from 14 HLHS patients, including 9 left ventricle, 5 right ventricle, 4 corresponding PBMC and 2 corresponding outflow tract samples (Table 1). Specimens were obtained from Vanderbilt Pediatric CHD Biorepository. The study was approved by Vanderbilt Institutional Review Board, and written informed consent was obtained from all heart tissue and blood sample donors. Blood samples were collected in BD Vacutainer® EDTA tubes (Becton, Dickinson and Company, NJ) prior to heart transplantation. Heart tissues were collected immediately after explant and were flash frozen in liquid nitrogen. Blood and tissues were stored in −80°C till used for DNA extraction. Patients had a confirmed diagnosis of HLHS at the time of tissue sample collection. DNA was extracted from these specimens using Qiagen DNeasy Blood & Tissue Kit Kit (Qiagen, Cat No. 69504) according to Manufacturer's instructions.

Sequencing of *HAND1*

We amplified the *HAND1* gene from 14 ventricular DNA samples by Polymerase Chain reaction (PCR) using AmpliTaq Gold® 360 Master Mix (ThermoFisher Scientific). We designed 5 primer sets covering 1996 bps including the 3 exons as well as flanking intronic regions (Supplement 1). We performed bidirectional Sanger sequencing utilizing BigDye Terminator chemistry sequencing kit and Applied Biosystems DNA Analyzer.

Whole exome sequencing comparison of PBMC and LV tissue samples for somatic mutation analysis

We completed WES on 10 samples including 4 ventricular samples, 2 outflow tract samples and 4 PBMCs (Table 1). The WES was performed at the Vanderbilt Advanced Genomics Core Facility using Illumina HiSeq 2500 with 100× coverage. Data were processed through Illumina's CASAVA v1.8.2 pipeline. We conducted thorough quality control based on the multi-stage quality control protocol developed at Vanderbilt [23, 24]. No sequencing quality concerns were observed. Alignments were performed using BWA[25] against human genome reference UCSC HG19. We marked duplicates using Picard[26], then performed local realignment and recalibration using the Genome Analysis Toolkit (GATK)[27]. Somatic mutations were inferred using GATK's analysis pipeline. The results were further filtered based on GATK's best practice. Annotations of SNV were performed using ANNOVAR[28].

Confirmatory Sanger sequencing of whole exome sequencing variants

We identified 271 sequence variations between blood and left ventricle (Figure 2). These variants were further filtered, and 71 instances of discrepancy with a read depth > 4,0 were identified. This low-stringent cutoff was meant to increase the sensitivity of detecting a somatic mutation in our sample, at the cost of lowering specificity[29]. Further filtering

using a MAF<1% left only 24 instances of sequence discrepancy in 14 genes: *BRD4*, *DMRTB1, FLJ44313, GLTSCR1, GPR153, HNRNPCL1, IQSEC2, MMP17, OGFRL1, POTED, RERE, RP1L1, SAMD1* and *SETD8*. We performed confirmatory Sanger sequencing of these 24 variants. In addition, we performed Sanger sequencing on 40 other variants in 12 additional genes based on loose biological plausibility: *C16orf71, CES1, COL4A4, EP400, EVX2, IFNA1, MADCAM1, MUC4, NOTCH2, PIEZO1, ZNF676* and *ZNF845*. In total, these 64 variants were located in 26 genes, and covered by 26 primer sets (Data Supplemental 1).

Mutation screening of whole exome sequencing data

In total, WES identified 36,426 single nucleotide variants (SNV) in the 10 tissue samples from 4 patients (Figure 3; Table 2). We first focused our analysis on 20 genes previously implicated in HLHS or ventricular development (*BMP2, FOXC2, FOXL1*, *GJA1, HAND1, HEY1, HEY2, HUWE1, IRX4, JAG1, MED20, MLL2, MYH6, NKX2-5, NOTCH1, NOTCH2, PTCH1, TBX2, TBX5* and *USP44*)[8–14, 16, 30, 31]. After comparing our sequencing results to the reference sequence UCSC hg19 [32], we found 8 SNVs which led to nonsynonymous codon changes (Figure 3A). Only 1, c.C4662A substitution in *NOTCH1*, had MAF<1%. Sanger sequencing was performed to confirm this mutation (Figure 3B).

Results

HAND1 frameshift (A126fs) mutation, previously reported to be commonly associated with HLHS, is not present in HLHS ventricular samples

We sought to test whether mutations in *HAND1* are present in left ventricular tissues of HLHS patients, as previously reported (17). We used DNA isolated from fresh frozen ventricle samples from 14 HLHS patients (9 LV and 5 RV samples) as described above (Figure 1A (Table 1)). *HAND1* is a 3.3kb gene consisting of 2 exons (Figure 1B). First, we sequenced a 533-basepair (bp) region of exon 2 containing the previously reported frame shift (A126fs) mutation. The A126fs mutation was absent in the 14 HLHS ventricular samples (Figure 1C). To rule out possible mutations elsewhere in *HAND1*, we sequenced a 1996-bp region consisting of all exons (1 and 2), the flanking introns, and a portion of the intergenic regions from all 14 HLHS patients' ventricles, and found no sequence variant in *HAND1*. Finally, WES analysis of LV tissue from 4 of the HLHS patients did not identify a *HAND1* mutation (Figure 1D).

Whole exome sequencing of left ventricular tissue and peripheral blood failed to demonstrate somatic mutation in HLHS

We also investigated the contribution of somatic mutations to HLHS beyond *HAND1* at the exome wide level. We performed WES of 4 LV samples, 4 corresponding PBMC samples and 2 corresponding outflow tract (OFT) samples (Figure 2; Table 1). We then compared the sequences from each patient's LV and OFT samples with the same patient's PBMC samples. The initial analysis identified 271 single nucleotide variations (SNVs) of potential discrepancies between PBMCs and LV/OFT tissues; however, each variant was in an area of low sequence coverage, suggesting these may be sequencing artifacts. Indeed, only 71 of 271 potential discrepant variants met the relatively low stringent filtering criterion of a read

depth >4,0 for increased sensitivity (recent studies use read depth >20 for somatic mutation calls [33]). As discussed above, we did not identify a somatic mutation in *HAND1*. Of the 71 variants, 47 found in LV/OFT tissues but not in PBMCs corresponded to known SNVs with a minor allele frequency (MAF) of >1%; therefore, we attributed these to erroneous variant calls in PBMC samples. Finally, we focused on the remaining 24 candidates that had a MAF <1%. To validate the initial WES results, we performed Sanger sequencing of 24 putative discrepant variants in 14 genes: *BRD4*, *DMRTB1, FLJ44313, GLTSCR1, GPR153, HNRNPCL1, IQSEC2, MMP17, OGFRL1, POTED, RERE, RP1L1, SAMD1* and SETD8 (Supplementary Data 1). We also performed Sanger sequencing on 40 additional potential discrepant variants in 12 genes based on novelty and predicted pathogenicity, and biological plausibility even though they did not meet the above, low-stringent filtering criteria: *C16orf71, CES1, COL4A4, EP400, EVX2, IFNA1, MADCAM1, MUC4, NOTCH2, PIEZO1, ZNF676 and ZNF845*. In summary, our Sanger sequencing of the 64 potential discrepant variants in 26 genes showed 100% match between blood and ventricular tissue, confirming our suspicion that the initial discrepancies were due to erroneous variant calls at low read depths. We detected no somatic mutations in the HLHS cases examined.

Mutation Screening of HLHS samples identified a novel *NOTCH1* truncation

From the WES of tissue sample of 4 HLHS patients, we identified total of 36,426 SNV calls, which are under ongoing further investigation. Of these, we focused on 20 genes previously implicated in HLHS or ventricular development (8–14, 16, 28, 29): *BMP2, FOXC2, FOXL1, GJA1, HAND1, HEY1, HEY2, HUWE1, IRX4, JAG1, MED20, MLL2, MYH6, NKX2-5, NOTCH1, NOTCH2, PTCH1, TBX2, TBX5* and *USP44*, and found 41 potential single nucleotide variants of which 8 were nonsynonymous changes (Figure 3A; Table 2). Of these, only 1 had a minor allele frequency (MAF) <1%. This variant, found in Patient 111 (Table 1), is a novel C to A substitution at position c.4662 (c.C4662A) of *NOTCH1* gene, which produces a premature stop codon replacing cysteine-1554 (p.C1554stop) in a Lin/NOTCH repeat (LNR) region of the extracellular domain (Figure 3C). Bidirectional sequencing confirmed that Patient 111 was heterozygous for this novel mutation, which appeared to be germline-derived based on sequencing of the patient's LV, OFT, PBMCs and his parent's DNA isolated from PBMCs (Figure 3B). We identified the same heterozygous mutation in the patient's father, though he is not affected by CHD. Interestingly, the patient's deceased paternal aunt was known to have a hypoplastic right ventricle; however, we did not have access to her tissue (Table 1).

Discussion

Increasing evidence supports a strong genetic component to HLHS pathogenesis, but despite extensive sequencing, the etiology remains unknown. Somatic mutations are implicated in atrial fibrillation and a variety of CHD [34, 35]; however, these results have not been reproducible. According to Reamon-Buettner et al, a recurrent somatic mutation in the *HAND1* gene was found in the majority (24 of 31) of the HLHS LV samples they studied [17]. Although this finding was refuted by Esposito et al [18], the latter study only examined right ventricle tissues, leaving open the crucial possibility of a somatic mutation within the developing left ventricle. Here, in examination of 14 HLHS ventricular samples, we found

no evidence of somatic nor germline mutation in *HAND1.* We note that, in the study by Reamon-Buettner and colleagues, DNA was obtained from ventricles fixed in formalin whereas our DNA samples were prepared from flash frozen tissue freshly obtained at the time of cardiac surgery[17, 34]. Since formalin fixation is known to cross link cytosine nucleotides, typically resulting in C to T (G to A) transitions [36], and fresh frozen tissue sequencing results are often contradictory [37, 38], we speculate that the earlier findings represent sequencing artifact due to formalin fixation of tissue samples. Our finding raises caution over claims of mutations based on sequencing from formalin fixed tissue samples. To our knowledge, this study is the first attempt to look for potential somatic mutations in hypoplastic LV tissues beyond HAND1. Our whole exome wide search for a somatic mutation in matched LV tissue samples from 4 HLHS patients failed to identify a somatic mutation. Given our small sample size, this study does not rule out a potential role of somatic mutations in pathogenesis of HLHS, but it suggests that recurrent mutations in HAND1 are not frequently involved in HLHS.

In one of he 14 HLHS patients, WES identified a novel, germline, nonsense mutation (c.C4662A) in *NOTCH1*, which encodes a transmembrane receptor involved in cell fate decisions. The variant carrier (patient 111) was heterozygous for this mutation, which replaces a stop codon for cysteine-1554. Since the predicted mutant protein lacks the entire transmembrane and cytoplasmic domains (Figure 3), it is highly likely that the truncated Notch1 is nonfunctional or pathogenic. The index patient's father, who does not have significant CHD, also carried this mutation in heterozygous state, indicating variable penetrance. While most previously reported HLHS cases are not associated with a *NOTCH1* mutation [22], our study strengthens the growing association of *NOTCH1* mutations and HLHS [14, 22, 30, 31].

Interestingly the patient's paternal aunt (now deceased) was diagnosed with hypoplastic right heart syndrome (HRHS), and had undergone palliative surgery as an infant. To our knowledge, this is the first report of a *NOTCH1* mutation associated with familial cases of a single ventricle defect, a spectrum of severe congenital heart defects including HLHS and HRHS, which are characterized by only one adequate-sized functional ventricle. A key unanswered question in HLHS pathophysiology is whether underdeveloped LV is a primary defect in LV morphogenesis or secondary to OFT abnormalities such as obstruction. Growing association of *NOTCH1* mutations with both OFT abnormalities and HLHS suggests a common pathogenesis, with modifier genes playing an important role. Our finding that a common *NOTCH1* mutation may be associated with both HLHS and HRHS lend support to the notion that, in some cases, defective OFT morphogenesis, rather than defective ventricular morphogenesis, may be primarily responsible for single ventricle defects. Future studies may provide insight by measuring expression of NOTCH1 and its downstream molecules at different regions within the heart, including the left ventricle, right ventricle and outflow tract.

HLHS was universally fatal until multi-staged palliative surgery began to prolong survival within the past two decades, though morbidity and mortality remain high. In recent years, two experimental approaches have been developed to treat HLHS. The first, fetal aortic valvuloplasty, was developed with the hope that alleviating outflow tract obstruction during

fetal development will improve outcome[39]. However, significant portion of patients fail to respond to this very high-risk procedure, and current guidelines that rely only on fetal echocardiographic features are ineffective in predicting therapeutic response[40]. An alternative approach, based on the premise that intrinsic deficit in cardiomyocyte proliferation is responsible for HLHS, involves transfer of cardiac progenitor cells to hypoplastic heart [41,42]. Further investigation into genetic causes of HLHS, including the role of critical modifier genes, as well as the induced pluripotent stem cell-based models of HLHS [21], are needed to differentiate potential myocyte intrinsic and extrinsic etiology. In summary, a better understanding of HLHS pathophysiology at the genetic and cell biological levels will herald a genome-informed individualized treatment plan for HLHS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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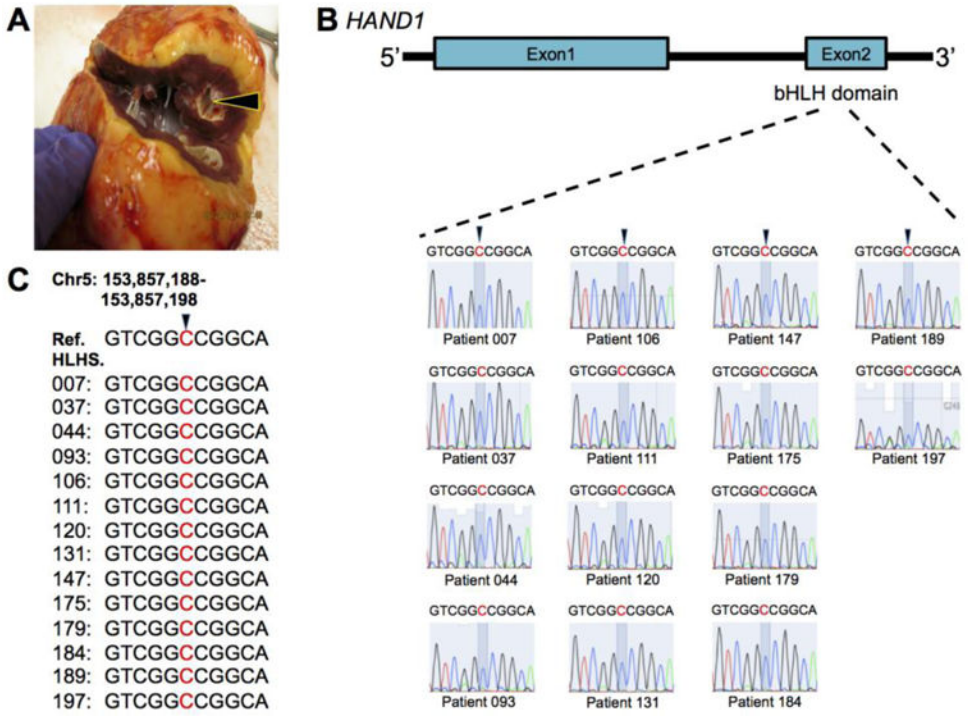
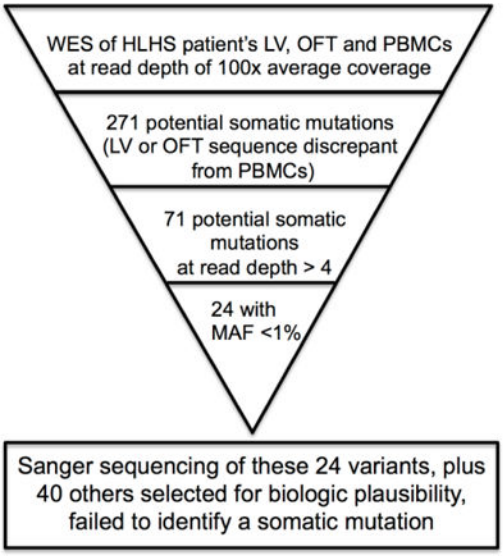


Figure 1. No mutation was found in *HAND1* in left ventricle of HLHS patients

(A) Using the Vanderbilt Congenital Heart Disease Tissue Bank, we isolated fresh frozen left ventricular (LV) and right ventricular (RV) tissue from 14 HLHS patients. (B) We amplified and sequenced a 1996 bp segment including *HAND1*'s entire coding region and a majority of the noncoding region. This included the Exon 1, which encodes the bHLH domain, previously reported to be the site of a recurrent somatic mutation (A126fs) in 24 of 31 HLHS LVs examined (17). (C) Shown are sequences corresponding to A126 in 9 HLHS LV and 5 HLHS RV samples. There were no mutations detected in this region or elsewhere in the *HAND1* coding region.



Tissue Samples Compared	# of sequence discrepancies	Sequence depth >4 reads	MAF <1%
Patient 004 LV vs PBMC	37	11	4
Patient 004 OFT vs PBMC	48	18	6
Patient 106 LV vs PBMC	48	10	3
Patient 106 OFT vs PBMC	43	9	3
Patient 007 LV vs PBMC	49	14	7
Patient 111 LV vs PBMCS	46	9	1
TOTAL	271	71	24

Figure 2. Exome wide search for somatic mutation in four HLHS patients

Right, Schema for validation of putative somatic mutations identified by WES. We examined 10 tissue samples including 4 patients' LVs and PBMCs, and additional outflow tract (OFT) tissue in 2 of those patients. WES at the 100× coverage identified 271 sequence discrepancies between LV or OFT and the corresponding PBMCs. All were in areas of low sequence coverage. 71 discrepant sequences had a read depth > 4,0. Of these, only 24 had MAF <1%. Sanger sequencing of these 24 samples failed to confirm a somatic mutation. We also conducted Sanger sequencing on 40 additional discrepant variants based on biological plausibility (see text), and found no somatic mutation. **Left**, breakdown of WES results by patients.

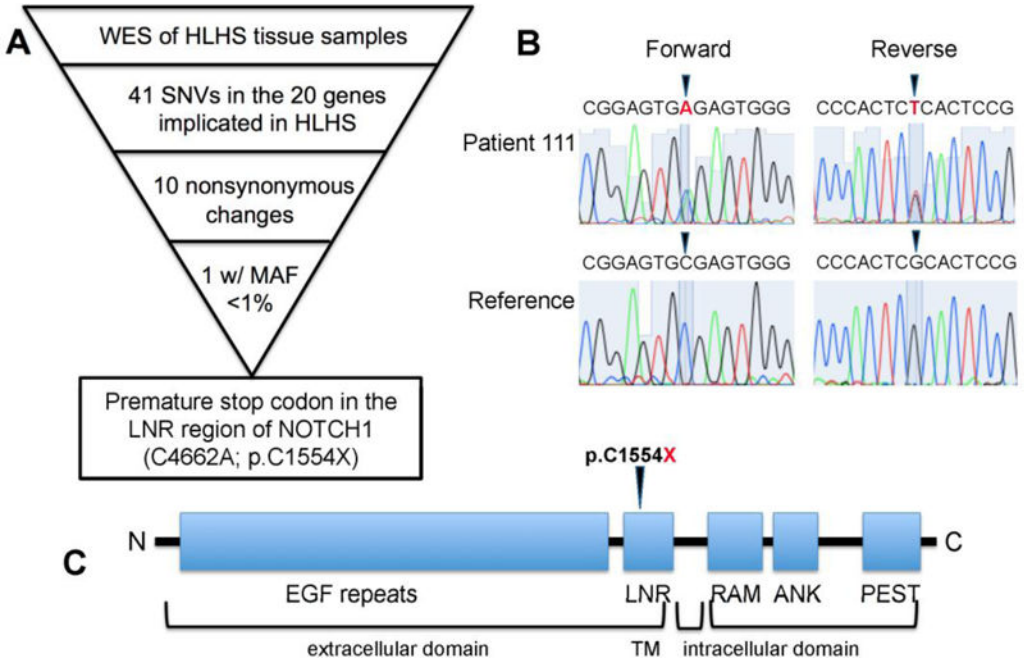


Figure 3. Search for mutations in genes previously implicated in HLHS reveals a novel truncation mutation in *NOTCH1*

(A) In WES of 10 samples of HLHS patients' left ventricular tissue, outflow tract, and blood, we focused on 20 genes previously associated with to HLHS or ventricular development. We detected total of 41 SNVs (Table 2). Of these, 10 were nonsynonymous changes, and only 1 had a MAF <1%. This mutation was a premature stop codon in the *NOTCH1* gene. (B) Sanger sequencing of patient 111's PBMC confirmed the mutation, a heterozygous A to C transversion, creating a stop codon at C1554. (C) The predicted mutant Notch1 protein was truncated at the Lin/NOTCH repeat (LNR) region of the extracellular domain, hence lacking the transmembrane (TM) and intracellular signaling domains. Also shown are RBP J-associated molecule (RAM) domain, ankyrin (ANK) domain and PEST domain.

Table 1

Clinical characteristics of HLHS patients

Study ID	Birth Weight (Kg)	Gender (M/F)	Age at 1st procedure (Day)	Other congenital Disease	Family His of CHD (Y/N)	Tissue Sequenced
007	3.2	F	6	N	*Unknown	LV, PBMC (WES)
044	2.86	M	5	N	N	LV, OFT, PBMC (WES)
106	2.96	M	1	N	N	LV, OFT, PBMC (WES)
111	4.40	M	1	N	Y**	LV, PBMC (WES)
37	Unknown	M	12	N	N	RV *(HAND1)*
93	1.39	M	7	N	N	RV *(HAND1)*
120	2.84	M	5	N	unknown	RV *(HAND1)*
131	2.54	M	2	N	N	RV *(HAND1)*
147	2.24	M	4	N	N	RV *(HAND1)*
175	4.08	M	4	N	N	LV *(HAND1)*
179	3.02	F	6	N	N	LV *(HAND1)*
184	3.70	M	4	N	N	LV *(HAND1)*
189	2.86	F	8	N	unknown	LV *(HAND1)*
197	2.55	M	2	N	N	LV *(HAND1)*

HLHS patient's clinical features, family history, and tissue analyzed are listed.

*Maternal cousin with unexplained childhood death.

**Paternal aunt with HRHS. CHD, congenital heart disease; LV, left ventricle; OFT, outflow tract; PBMC, peripheral blood mononuclear cells; WES, whole exome sequencing; *HAND1*, targeted sequencing of *HAND1* gene.

Table 2

Germline SNVs in the 20 genes implicated in HLHS and/or ventricular development, identified by WES.

Genes Implicated in HLHS	SNV identified	Chromo some	Location	Reference sequence	Nucleotide detected	Type of change	MAF
BMP2	Yes	20	6750882	T	G	nonsynonymous SNV	0.03
		20	6751034	A	G	synonymous SNV	
		20	6759115	A	T	nonsynonymous SNV	0.23
FOXC2	No						
FOXL1	No						
GJA1	No						
HAND1	No						
HEY1	No						
HEY2	Yes	6	126080522	G	C	synonymous SNV	
HUWE1	Yes	X	53563589	A	G	synonymous SNV	
		X	53641747	T	C	synonymous SNV	
IRX4	Yes	5	1878212	G	A	synonymous SNV	
		5	1878440	C	T	synonymous SNV	
		5	1880865	T	C	synonymous SNV	
		5	1880891	C	T	nonsynonymous SNV	0.13
		5	1882129	T	G	synonymous SNV	
JAG1	Yes	20	10620275	G	A	synonymous SNV	
		20	10620386	A	G	synonymous SNV	
		20	10625804	T	G	synonymous SNV	
		20	10632861	G	A	synonymous SNV	
		20	10633237	G	A	synonymous SNV	
		20	10637057	T	C	synonymous SNV	
		20	10653469	C	T	synonymous SNV	
MED20	No						
MLL2	Yes	12	49424534	G	A	synonymous SNV	
		12	49425978	T	C	synonymous SNV	
		12	49427652	C	T	synonymous SNV	

Genes Implicated in HLHS	SNV identified	Chromo some	Location	Reference sequence	Nucleotide detected	Type of change	MAF
		12	49431410	G	A	synonymous SNV	
		12	49434074	C	A	synonymous SNV	
		12	49444545	G	A	synonymous SNV	
		12	49445028	G	A	nonsynonymous SNV	0.015
MYH6	No						
NKX2-5	Yes	5	172662024	T	C	synonymous SNV	
NOTCH1	Yes	9	139391636	G	A	synonymous SNV	
		9	139395085	G	A	synonymous SNV	
		9	139397707	G	A	synonymous SNV	
		9	139399481	G	T	stopgain SNV	Novel
		9	139407932	A	G	synonymous SNV	
		9	139418260	A	G	synonymous SNV	
NOTCH2	Yes	1	120458004	A	T	synonymous SNV	
		1	120611960	C	T	nonsynonymous SNV	0.14
		1	120611964	G	C	nonsynonymous SNV	0.45
PTCH1	Yes	9	98209594	G	A	nonsynonymous SNV	0.30
		9	98212141	G	A	synonymous SNV	
		9	98238358	G	A	synonymous SNV	
		9	98238379	A	G	synonymous SNV	
TBX2	Yes	17	59477903	C	A	synonymous SNV	
		17	59485555	C	T	synonymous SNV	
TBX5	No						
USP44	No						

SNV, single nucleotide variant; MAF, minor allele frequency